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Nucleotide Sequence Analysis of a Variable Region of the Large Subunit rRNA for Identification of Marine-Occurring Yeasts

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Abstract. A 230-nucleotide region of the large subunit (LSU) ribosomal RNA was examined to determine whether signature nucleotide sequences could be used for species identifications of basidiomycetous yeasts. Multiple strains of genetically defined heterothallic species of *Rhodosporidium*, *Leucosporidium*, *Cystofilobasidium*, and *Sporidiobolus* demonstrated that nucleotide sequences within these species are homologous and that differences between species range from 1 to 20 or more bases. Also included in this study were several homothallic species of these teleomorphic genera and some anamorphs assigned to *Rhodotorula* and *Candida*. Those results indicated close relationships among certain homothallic species, particularly in the genus *Mrakia*, and potential relationships of homothallic and anamorphic strains to several teleomorphs. The data suggest that LSU sequences can be used for yeast identifications with the possible exception of closely related homothallic species.

The ability to identify and quantify microeukaryotes in marine and aquatic systems has been a long-standing problem in developing knowledge of microbially based food webs. These and other problems in environmental biology can be approached with tools that are rapidly being developed in molecular biology. For example, nucleotide sequencing of ribosomal RNA (rRNA) was suggested for the characterization of organisms of uncertain affinity [10], and the concept was advanced by the use of specific oligonucleotide probes for the identification of individual cells of bacteria and yeasts [2].

The potential use of rRNA sequence analysis as an environmental tool stems from its successful employment in phylogenetic studies. Initially, analyses of the 5S fraction [19, 20] revolutionized the understanding of prokaryotic evolution. Subsequently, owing to the small size of the 5S fraction and corresponding limited information, phylogenetic studies shifted to the small and large subunits of rRNA (SSU and LSU). The large subunit has particular interest due to the presence of both highly conserved and variable regions. Primary and secondary structures in the conserved regions are sufficiently homologous to depict evolutionary relationships among the eukaryotes [14, 15]. The variable regions, of which there are 18, are of interest because their size variations (1–873 nucleotides) have been sustained, within phylogenetic groups, throughout the course of evolution [16]. One of these regions, designated V3 [16], is sufficiently conserved to demonstrate phylogenetic differences among genera and species within specific groups of organisms such as ascomycetous and basidiomycetous fungi [5–7].

The present study was to determine whether species of eukaryotes can be identified by specific oligonucleotide sequences in the V3 region. To accomplish this objective, we examined closely related species of teliospore-producing basidiomycetous yeasts that had been genetically characterized on the basis of mating incompatibility systems [3]. Nine heterothallic species, with several strains/species, were examined to determine sequence variation within and between genetically isolated species. To further gauge the extent of variability and reliability of sequence specificity, we examined 13 closely and distantly related species that lack apparent sexual incompatibility systems.

The teliosporic yeasts, which consist of five genera, have a widespread occurrence, particularly in marine and polar habitats. Generic separations are based on morphological and biochemical characteristics. *Sporidiobolus* and *Rhodosporidium* were the first two genera described. Both genera are red pig-

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SPECIES	STRAIN #	SEX	364		400 – 440	460	479
Rm diobovatum	ML 2253	A2	AAGAUGAAAAGCACUUUGGAAAGAGUU		AACAGUACGUGAAAUUGUUGGAAGGGAAACGCUUGAAGUCAGACUUGCUUGCCG		GAGCUUGCUUC
	CBS 6084	A2	---------------------------		--		-----------
	ML 2966	A2	---------------------------		--		-----------
	ML 2346	A1	---------------------------		--		-----------
	CBS 6085 T	A1	---------------------------		--		-----------
R graminis	Y-2474 T	NS	---------------------------		--		-----------
R glutinis v glutinis	Y-2502 T	NS	---------------------------		--		-----------
Rm sphaerocarpum	CBS 5939 T	A2	---------------------------		--		- --AAC-
	CBS 5941	SS	---------------------------		--		- --AAC-
	ML 2599	A2	---------------------------		--		- --AAC-
R glutinis v dairenensis	CBS 4406 T	NS	---------------------------		--		---AA --
R rubra	Y-1592 T	NS	---------------------------		--		---AA --
R mucilaginosa	CBS 316	NS	---------------------------		--		---AA --
Rm toruloides	CBS 14 T	A1	---------------------------		--A---		- ----N-- -
	CBS 349	A2	---------------------------		--A---		- ---------
Rm paludigenum	ML 2226	A1	---------------------------		--		-----------
	Y-12923	A2	---------------------------		--		-----------
S microsporus	CBS 7041	SS	---------------------------		--UU--		-N-U--AN-C-
Rm fluviale	ML 1590 T	SS	---------------------------		--UU--		-G-U--A--C-
S ruinenii v ruinenii	CBS 4999	SS	---------------------------		---AUU--		-G-------C-
	Y-12930 T	SS	---------------------------		---AUU--		-G----N--C-
S ruinenii v coprophilus	Y-12938	SS	---------------------------		---AUU--		-G-------C-
S salmonicolor	ML 2242	A1	---------------------------		---AUU--		------N----
Rm dacryoidum	CBS 6354	A1B2	---------------------------		--------------------A-----------------------G---G- A-		UGCU-N-NG-G
	CBS 6355	A2B1	---------------------------		--------------------A-----------------------G---G- A-		UGCU---GG-G
L scottii	Y-1497 T	A5B1	---------------------------	A	---AUU--	GAGUUCAGCCGCAAG	-U-UA-U----
	CBS 5931	A1B1	-----------N---------------	A	---N---AUU--	GAGUUCNGCCGCAAG	-U-UA-U----
M gelidum	Y-7211 T	SS	-----------------N---------	N	-------------------AA----------A----------U-N-----U--N	AGA CUCAGCCGAUUCUGUCG	-N-UACUUC--
M nivalis	Y-7204 T	SS	-----------------N---------	N	-------------------AA----------A----------U-N-----U--N	AGA CUCAGCCGAUUCUGUCG	-N-UACUUC--
M frigidum	Y-7202 T	SS	-----------------N---------	N	------N------------NA----------A----------U-N-----U--N	AGA CUCAGCCGAUUCUGUCG	-N-UACUUC--
C bisporidii	CBS 6347	A2B2	--------------------------C	A	--------------------A----------A----------UNG---C----U	AGA UUCAGCC UUCUN	-U-UA-UUC-A
	CBS 6346 T	A1B1	-----------N--------------C	A	------N-------------A----------A----------UNG---C----U	AGA UUCAGCC UUCUN	-U-UN-UUC-A
C capitatum	CBS 6358 T	SS	--------------------------C	A	--------------------A----------A----------U-G---C----U	AGA UUCAGCC UUCUN	-U-UA-UUC-A
C infirmominiatum	CBS 6352	A3	-----------N--------------C	A	--------------------A----------A----------U-G---C----U	AGA UUCAGCC UUCUN	-U-UA-UUC-A
	Y-1586 T	A1	--------------------------C	A	--------------------A----------A----------U-G---C----U	AGA UUCAGCC UUCUG	-U-UA-UUC-A
L lari-marini	CBS 7420 T	SS	--------------------------C	A	--------------------A----------A----------U-G---C----U	AGA UUCAGCC UUCUG	-U-UA-UUC-A
Ca humicola	CBS 2042	NS	---------------------------	A	--------------------A----------A----------U-G-- --CAUU	GGA CUCAGCCGGUUUU CG	-U-UA-UUCCU

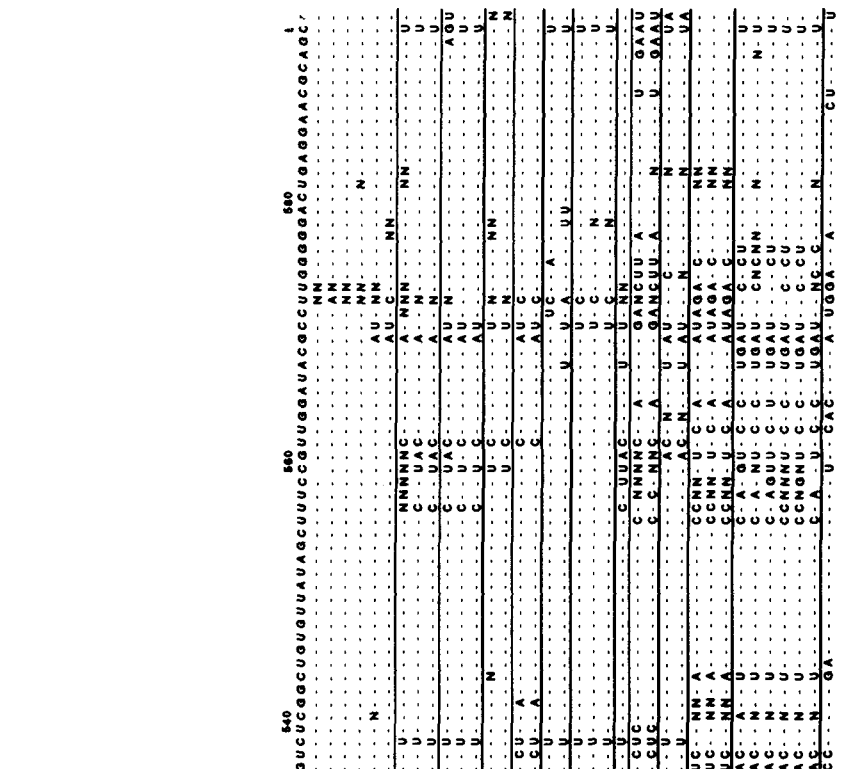

Fig. 1. Alignment of LSU rRNA partial sequences from species of basidiomycetous yeasts. The sequence of *Rhodosporidium diobovatum* ML 2253 serves as a reference; dashes indicate nucleotides identical to the reference strain; blanks indicate no bases at that position. N: an unidentified base. Genus abbreviations: C, *Cystofilobasidium*; Ca, *Candida*; L, *Leucosporidium*; M, *Mrakia*; R, *Rhodotorula*; Rm, *Rhodosporidium*; S, *Sporidiobolus*. Strain abbreviations: ML, RSMAS culture collection; CBS, Centraalburea voor Schimmelcultures; Y, ARS culture collection (NRRL); T, type strain. Sex: A1, B1, etc, heterothallic mating type; SS, homothallic teliospore formation; NS, anamorphic strain.

mented; *Sporidiobolus* differs by the presence of ballistospores. *Cystofilobasidium*, another red-pigmented genus, was separated from *Rhodosporidium* [13] on the ability to utilize inositol and the presence of Q8 ubiquinones, a septate basidium, and a primitive dolipore that lacks vesicles. The genus *Leucosporidium* was described to encompass a group of low-temperature white yeasts from polar regions. Several species of *Leucosporidium* were placed in the genus *Mrakia* [21], with the characteristics of the ability to ferment various carbon compounds, a Q9 ubiquinone system, and a homokaryotic teliospore that produces a holometabasidium.

These teliosporic yeasts have mating systems that may consist of two mating types within a species (a unifactorial, biallelic incompatibility system) or they may have multiple mating types (unifactorial, multiple allelic, or bifactorial systems). Some strains lack a heterothallic mating system and produce sexual or sexual-like teliospores by homothallic mechanisms. Most basidiomycetous yeasts, when isolated from nature, are in the haploid state, and in many cases the strains have lost the ability to reproduce sexually. Such strains are taxonomically placed in anamorphic genera. The anamorphic (teleomorphic) states are: *Rhodotorula* (*Rhodosporidium*, *Cystofilobasidium*), *Candida* (*Leucosporidium*, *Mrakia*), *Sporobolomyces* (*Sporidiobolus*). These are not limited relationships; for example, there are in excess of 30 species of basidiomycetous candidas [11] whose relationships are unknown and may encompass a wide range of phylogenetic groups.

Materials and Methods

RNA extraction. Strains are listed in Fig. 1. Cells were grown at 19°C in 50 ml of YM medium [6] on a rotary shaker at 150 rpm for 16–24 h. Exceptions were low-temperature organisms (*Mrakia* spp.) that were grown at 15°C. Cells were harvested by centrifugation, suspended in buffer (1 g wet wt/10 ml 4 *M* guanidinium thiocyanate), and broken in a Braun cell homogenizer. Cell debris was removed by centrifugation, and 0.5% 5 *M* NaCl was added to the supernatant. RNA was purified by repeated dissolution and centrifugation in phenol/chloroform/isoamyl alcohol (25 : 24 : 1 pts) followed by precipitation in cold ethanol, drying with acetone, dissolving in pH 7.5 20 m*M* Tris with 10% pH 6.0 3 *M* sodium acetate and precipitation in cold ethanol.

Sequence reaction and analysis. Sequencing of rRNA used specific oligonucleotide primers and the dideoxy nucleotide chain terminating method [10, 18]. The primer used was 5'GGTCCGTGTTT CAAGACG, representing position 637–654 in *Saccharomyces cerevisiae* [8]. Nucleotide fragments generated in the chain terminating reactions were separated on 8% acrylamide-8 *M* urea gels and visualized with autoradiography. Sequences were manually aligned.

Results and Discussion

Critical to understanding sequence specificity is the determination of differences or homogeneity among strains of a species. Sequence analyses (Fig. 1) of multiple strains of *Rhodosporidium diobovatum*, *R. sphaerocarpum*, *R. paludigenum*, *R. dacryodium*, *R. toruloides*, *Leucosporidium scottii*, *Cystofilobasidium bisporidii*, and *C. infirmominiatum* demonstrate homologous sequences within species. There are distinct differences between these heterothallic. The most obvious distinction is the aligment gap insertion at 447–468. Species with the gap belong to the sexual genera *Rhodosporidium* and *Sporidiobolus*, whose generic separation is based on the ephemeral characteristic of ballistospore production [9]. Three of the heterothallic species (*Rhodosporidium toruloides*, *R. sphaerocarpum*, *R. diobovatum*) are taxonomically related as members of the "*R. glutinis* group" of species; *R. glutinis* is an environmentally ubiquitous anamorphic species characterized by the ability to assimilate NO3 and the carbon compounds maltose, melezitose, and raffinose, but not melibiose or erythritol. The species is genetically diverse, as indicated by G + C values that range from 60 to 68 mol% [12]. The three sexual species included in this "*R. glutinis* group" can be distinguished by nucleotide sequences at 522–528. Similarly, the teleomorphic species *Rhodosporidium paludigenum*, *R. dacryoidum*, and *Sporidiobolus salmonicolor* can be differentiated in this same region.

The homothallic species *Sporidiobolus ruinenii* and *Rhodosporidium fluviale* have distinct nucleotide sequences at 506–528. The two varieties of *S. ruinenii* have homologous sequences; varietal status is based on 63% DNA relatedness and differences in mode of teliospore formation [9]. *Sporidiobolus microsporus*, a species that has been cited [1] but never formally described, differs from *R. fluviale* at 568–580. The two species are similar in their taxonomic characteristics [1, 4], differing in their ability to utilize alpha glucosides as sole sources of carbon.

Among the anamorphic species examined were two varieties of *R. glutinis* (Y-2502, ML 294) that were dissimilar in several regions; this emphasizes the heterogeneity of the "*R. glutinis* group." The type strains of *R. glutinis* var. *glutinis* (Y-2502) and *R. graminis* (Y-2474) have a difference of one base at position 519 and a 30% DNA relatedness [9]. Both strains have a nucleotide sequence similar to *R. diobovatum*, differing by two bases at 570. The strain of *R. glutinis* var. *diarenensis* has a nucleotide se-

quence pattern similar to *R. sphaerocarpum* but differs at 476, 509, and 594. *Rhodotorula rubra* and *R. mucilaginosa* (a synonym of *R. rubra*) are also similar to *R. sphaerocarpum*, differing at 476, 509, and 561.

Species with nucleotides at 447–468 fall into 4 groups, which can be differentiated in that region and through major portions of 479–595. *Leucosporidium scottii* and *Mrakia* spp. have distinct nucleotide sequences, in agreement with their morphological and physiological differences. The three species of *Mrakia* lack a sexual incompatibility system; therefore, species separation is arbitrary and has been based on various biochemical tests. Opinions differ on species definitions; for example, the recommendation was made to place *M. nivalis* in synonymy with *M. frigidum* [22]. The homologous sequences affirm the close relationships of the strains.

Cystofilobasidium has an analogous situation to the "*R. glutinis* group." The three species of *Cystofilobasidium* were originally classified as strains of *Rhodotorula infirmominiata*, from which two heterothallic and one homokaryotic species were described. Individual species of *Cystofilobasidium* can be distinguished by sequence differences at 410–578. Included in this figure with *Cystofilobasidium* is *Leucosporidium larimarini*, a recently described species [17] whose method of teliospore germination and types of ubiquinone system and dolipore are unknown. The sequence similarity to *Cystofilobasidium* spp. suggests that this species may be misclassified.

Candida humicola is a basidiomycetous species of *Candida*. The mode of reproduction and relationship to teliospore-forming yeasts are unknown, and the species was included for comparative purposes. The nucleotide sequence at 483–601 is distinct from the other species studied, suggesting that other unrelated species have similarly distinct sequence patterns.

The use of LSU rRNA nucleotide sequences for phylogenetic separation of fungi was established by the examination of eight species of *Fusarium* [5]. Based on the number of nucleotide position differences, evolutionary trees were developed, with the conclusion that sequence analysis can be used for rapid determination of strain classification and relationship of species within genera. In a separate study, the sequence similarities of portions of the SSU and LSU rRNA were compared for 13 species of ten genera of heterobasidiomycetous yeasts [7]. Those results, which also emphasized phylogenetic relationships based on position differences, indicated that genera, in addition to species, appear to be characterized by unique nucleotide sequences signatures. Specifically, among the teleomorphic yeasts, *L. scottii*, *S. johnsonii*, and *R. toruloides* could be individually recognized in the LSU by several base substitutions and by an alignment gap.

Our present study, which suggests certain taxonomic relationships among species, does not consider phylogeny; instead, our focus is on species-specific nucleotide sequences. The results demonstrate that nucleotide sequences of strains within a species are homologous and that sequences of up to 20 or more bases are unique to certain species or groups of species. Close related species, such as *Cystofilobasidium* spp., may have sequence differences limited to one to four bases. In the genus *Mrakia*, where nucleotide sequences are identical and a heterothallic mating system is lacking, species separations will require verification through DNA : DNA hybridizations.

Our results demonstrate that LSU sequences are specific for the reliable identification of all but possibly the most closely related species. This reliability may be improved by examination of other LSU variable regions. With the recent advances in non-radioactive probes [2], the potential is raised for the widespread use of the LSU for developing oligonucleotide probes for rapid and accurate identification of yeasts and other eukaryotes in environmental sciences.

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